Lipid Emulsion Containing High Amounts of n3 Fatty Acids (Omegaven) as Opposed to n6 Fatty Acids (Intralipid) Preserves Insulin Signaling and Glucose Uptake in Perfused Rat Hearts

Lou, Phing-How ; Lucchinetti, Eliana ; Hersberger, Martin ; Clanachan, Alexander S ; Zaugg, Michael

Abstract: BACKGROUND: It is currently unknown whether acute exposure to n3 fatty acid–containing fish oil–based lipid emulsion Omegaven as opposed to the n6 fatty acid–containing soybean oil–based lipid emulsion Intralipid is more favorable in terms of insulin signaling and glucose uptake in the intact beating heart. METHODS: Sprague–Dawley rat hearts were perfused in the working mode for 90 minutes in the presence of 11 mM glucose and 1.2 mM palmitate bound to albumin, the first 30 minutes without insulin followed by 60 minutes with insulin (50 mU/L). Hearts were randomly allocated to 100 µM Intralipid, 100 µM Omegaven, or no emulsion (insulin treatment alone) for 60 minutes. Glycolysis and glycogen synthesis were measured with the radioactive tracer [5-3H]glucose, and glucose uptake was calculated. Phosphorylation of protein phosphatase 2A (PP2A), protein kinase Akt, and phosphofructokinase (PFK)-2 was measured by immunoblotting. Glycolytic metabolites were determined by enzymatic assays. Mass spectrometry was used to establish acylcarnitine profiles. Nuclear factor B (NF B) nuclear translocation served as reactive oxygen species (ROS) biosensor. RESULTS: Insulin-mediated glucose uptake was decreased by Intralipid (4.9 ± 0.4 vs 3.7 ± 0.3 mol/gram dry heart weight [gdw] · min; P = .047) due to both reduced glycolysis and glycogen synthesis. In contrast, Omegaven treatment did not affect insulin-mediated glycolysis or glycogen synthesis and thus preserved glucose uptake (5.1 ± 0.3 vs 4.9 ± 0.4 mol/gdw · min; P = .94). While Intralipid did not affect PP2A phosphorylation status, Omegaven resulted in significantly enhanced tyrosine phosphorylation and inhibition of PP2A. This was accompanied by increased selective threonine phosphorylation of Akt and the downstream target PFK-2 at S483. PFK-1 activity was increased when compared with Intralipid as measured by the ratio of fructose 1,6-bisphosphate to fructose 6-phosphate (Omegaven 0.60 ± 0.11 versus Intralipid 0.47 ± 0.09; P = .023), consistent with increased formation of fructose 2,6-bisphosphate by PFK2, its main allosteric activator. Omegaven lead to accumulation of acylcarnitines and fostered a prooxidant response as evidenced by NF B nuclear translocation and activation. CONCLUSIONS: Omegaven as opposed to Intralipid preserves glucose uptake via the PP2A–Akt–PFK pathway in intact beating hearts. n3 fatty acids decelerate -oxidation causing accumulation of acylcarnitine species and a prooxidant response, which likely inhibits redox-sensitive PP2A and thus preserves insulin signaling and glucose uptake.

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ORIGINAL LABORATORY RESEARCH REPORT

Lipid Emulsion Containing High Amounts of n3 Fatty Acids (Omegaven) as Opposed to n6 Fatty Acids (Intralipid) Preserves Insulin Signaling and Glucose Uptake in Perfused Rat Hearts

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BACKGROUND: It is currently unknown whether acute exposure to n3 fatty acid–containing fish oil–based lipid emulsion Omegaven as opposed to the n6 fatty acid–containing soybean oil–based lipid emulsion Intralipid is more favorable in terms of insulin signaling and glucose uptake in the intact beating heart.

METHODS: Sprague–Dawley rat hearts were perfused in the working mode for 90 minutes in the presence of 11 mM glucose and 1.2 mM palmitate bound to albumin, the first 30 minutes without insulin followed by 60 minutes with insulin (50 mU/L). Hearts were randomly allocated to 100 µM Intralipid, 100 µM Omegaven, or no emulsion (insulin treatment alone) for 60 minutes. Glycolysis and glycogen synthesis were measured with the radioactive tracer [5-^{3}H]glucose, and glucose uptake was calculated. Phosphorylation of protein phosphatase 2A (PP2A), protein kinase Akt, and phosphofructokinase (PFK)-2 was measured by immunoblotting. Glycolytic metabolites were determined by enzymatic assays. Mass spectrometry was used to establish acylcarnitine profiles. Nuclear factor κB (NFκB) nuclear translocation served as reactive oxygen species (ROS) biosensor.

RESULTS: Insulin-mediated glucose uptake was decreased by Intralipid (4.9 ± 0.4 vs 3.7 ± 0.3 µmol/gram dry heart weight [gdw]·min; P = .047) due to both reduced glycolysis and glycogen synthesis. In contrast, Omegaven treatment did not affect insulin-mediated glycolysis or glycogen synthesis and thus preserved glucose uptake (5.1 ± 0.3 vs 4.9 ± 0.4 µmol/gdw·min; P = .94). While Intralipid did not affect PP2A phosphorylation status, Omegaven resulted in significantly enhanced tyrosine phosphorylation and inhibition of PP2A. This was accompanied by increased selective threonine phosphorylation of Akt and the downstream target PFK-2 at S483. PFK-1 activity was increased when compared with Intralipid as measured by the ratio of fructose 1,6-bisphosphate to fructose 6-phosphate (Omegaven 0.60 ± 0.11 versus Intralipid 0.47 ± 0.09; P = .023), consistent with increased formation of fructose 2,6-bisphosphate by PFK2, its main allosteric activator. Omegaven lead to accumulation of acylcarnitines and fostered a prooxidant response as evidenced by NFκB nuclear translocation and activation.

CONCLUSIONS: Omegaven as opposed to Intralipid preserves glucose uptake via the PP2A–Akt–PFK pathway in intact beating hearts. n3 fatty acids decelerate β-oxidation causing accumulation of acylcarnitine species and a prooxidant response, which likely inhibits redox-sensitive PP2A and thus preserves insulin signaling and glucose uptake. (Anesth Analg 2020;130:37–48)

KEY POINTS

- **Question:** How do lipid emulsions containing n3 fatty acids affect insulin signaling and glucose uptake in the intact beating heart?
- **Finding:** Using perfused rat hearts, we show that a lipid emulsion containing high amounts of n3 fatty acids (Omegaven) as opposed to n6 fatty acids (Intralipid) preserves insulin signaling, glycolysis, and glucose uptake possibly by inhibition of protein phosphatase 2A.
- **Meaning:** Omegaven compared to Intralipid does not induce insulin resistance and thus has more favorable metabolic effects in the beating heart.

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Millions of patients worldwide depend on total or partial parenteral nutrition containing lipid emulsions. Indications for parenteral nutrition with lipid emulsions include critically ill patients admitted to the intensive care unit, short- or long-term intestinal failure, severe malnutrition, cancer, pancreatitis, major trauma, burns, and certain surgeries.[1] In these large groups of patients requiring parenteral nutrition, mixtures containing lipid emulsions as opposed to exclusive high-carbohydrate solutions are preferred because a more physiological provision of calories can be achieved. However, lipid emulsions elicit insulin resistance and adverse metabolic effects on glucose metabolism,[2] particularly in already metabolically compromised patients.[3] These metabolic changes also affect cardiac function[4] and ultimately impair patient outcomes.[5]

Insulin resistance is a key feature of type 2 diabetic metabolism and is defined as impaired sensitivity to effects of insulin on whole-body glucose disposal. In the heart, it is defined as the reduced ability of the heart to increase glucose uptake in response to insulin stimulation.[6] Cardiac insulin resistance ultimately reduces cardiac performance.[7] Intralipid (Fresenius Kabi AG, Bad Homburg, Germany), a soybean oil–based product containing long-chain n6 fatty acids, is the most prevalently used lipid emulsion in medicine to date.[8] However, infusion of Intralipid, for example, as part of parenteral nutrition, impairs glucose uptake via the Randle cycle[9] and reduces insulin sensitivity in both healthy volunteers and diabetic subjects.[10,11] In contrast, lipid emulsions containing n3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3) may have beneficial effects on glucose metabolism and prevent lipid-induced insulin resistance.[12] While cell-based studies suggest beneficial effects of lipid emulsions with DHA and EPA such as Omegaven (Fresenius Kabi AG, Bad Homburg, Germany) on cardiac glucose metabolism,[13] it has never been tested in the intact beating heart whether Omegaven as opposed to Intralipid more favorably affects insulin-mediated glucose uptake, and a direct comparison of the cardiometabolic effects of these lipid emulsions is warranted. We hypothesized that acute administration of Omegaven as opposed to Intralipid would preserve glucose uptake in the heart in the presence of the fatty acid palmitate, a condition mimicking stress and promoting insulin insensitivity. Because previous reports in cells implicated inhibition of the serine/threonine protein phosphatase 2A (PP2A) in beneficial effects of polyunsaturated fatty acids on insulin signaling,[14] we further tested whether Omegaven would inhibit PP2A and enhance Akt signaling by phosphorylating and activating its downstream target phosphofructokinase (PFK)-2, and thus ultimately increase the activity of the rate-limiting enzyme of glycolysis, PFK-1.

METHODS

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and adheres to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (https://www.nc3rs.org.uk/arrive-guidelines). The experimental protocol used in this investigation was approved by the University of Alberta Animal Policy and Welfare Committee.

All materials were from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

Working Heart Perfusions and Protocols

Sprague–Dawley rats (14 weeks of age) were anesthetized with pentobarbital (150 mg/kg, intraperitoneally). Each heart was rapidly removed and perfused initially in a nonworking Langendorff mode with Krebs–Henseleit solution for 15 minutes. The working mode perfusion was subsequently established (11.5 mm Hg preload, 80 mm Hg afterload, 5 Hz) with a recirculating perfusate of 100 mL (37°C; pH, 7.4) gassed with 95% oxygen (O_2)/5% carbon dioxide (CO_2) mixture that consisted of a modified Krebs–Henseleit solution containing (mmol/L) KCl (4.7), NaCl (118), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25), glucose (11), palmitate (1.2, prebound to 3% bovine serum albumin), and no insulin for the first 30 minutes followed by a 60-minute perfusion with insulin 50 mU/L. Some hearts were perfused without insulin for 90 minutes. Cardiac output (CO; mL/min) and aortic flow (mL/min) were measured using ultrasonic flow probes (Transonic T206; Transonic Systems Inc, Ithaca, NY) placed in the left atrial inflow and the aortic outflow lines. Left ventricular work (LVW; mL/min·mm Hg) was calculated as $\mathrm{LVW} = \mathrm{CO} \times (\text{aortic systolic pressure} - \text{preload})$. Coronary flow (mL/min) was calculated as the difference between CO and aortic flow, and coronary vascular conductance (mL/min mm Hg) was computed as CVC = coronary flow × (mean aortic pressure – preload). Measurements of mechanical function were averaged for the initial 30 minutes of aerobic perfusion without insulin (baseline) in the perfusate and for the following 60 minutes of perfusion in the presence of 50 mU/L insulin (response to insulin). Hearts were randomly assigned to the following 4 groups (N = 7 per group; see Figure 1): (1) hearts perfused for 90 minutes without insulin (no-INS); (2) hearts perfused without insulin for 30 minutes followed by 60 minutes with insulin (INS); and (3 and 4) hearts treated with 100 µM Intralipid (INS/IL) or 100 µM Omegaven (INS/OV; Fresenius Kabi Switzerland AG, Oberdorf, Switzerland). Previous studies with total parenteral nutrition in rats showed plasma triglyceride concentrations in the range of 100–500 µM.[15] An

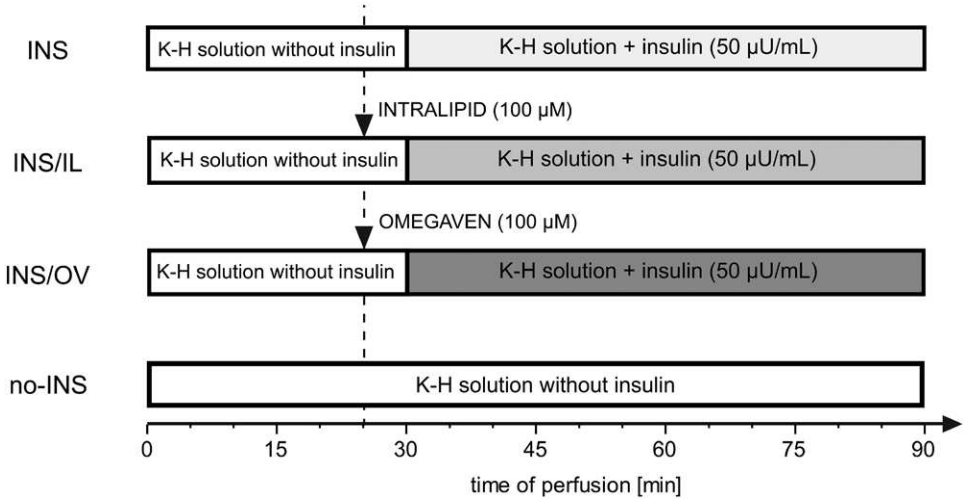

Figure 1. Experimental protocols. After a 15-min period of Langendorff (nonworking) stabilization period, the working mode was established. Hearts were randomly assigned to the following groups. no-INS: hearts perfused for 90 min without insulin (time-matched control); INS: hearts perfused for 30 min without insulin followed by 60 min with insulin (50 mU/L); INS/IL: hearts were perfused identical to INS hearts, but Intralipid was added 5 min before insulin administration; and INS/OV: hearts were perfused identical to INS hearts, but Omegaven was added 5 min before insulin administration. Arrows indicate timing of lipid emulsion administration. INS indicates insulin alone; INS/IL, perfusion with insulin plus Intralipid; INS/OV, perfusion with insulin plus Omegaven; K-H, Krebs–Henseleit solution buffer containing 11 mM glucose and 1.2 mM palmitate bound to albumin; no-INS, perfusion without insulin.

average molecular weight of 873 g/M was calculated for Intralipid and 913 g/M for Omegaven, respectively, based on the composition of the 2 emulsions as detailed in Supplemental Digital Content, Table 1, http://links.lww.com/AA/C857. The n6-to-n3 ratio for Intralipid is 7:1 and for Omegaven is 1:8. Intralipid contains α-linolenic acid (C18:3 n3), which shows limited conversion to EPA and DHA in mammals.[16] Additional hearts were perfused for 30 minutes to determine the glycogen content necessary for the calculation of the glucose uptake (see formula below). At the end of the perfusions, the left ventricles were collected and all hearts were immediately frozen in liquid nitrogen with Wollenberger clamps and stored at −80°C for subsequent molecular analyses.

Metabolic Flux Measurements of Glycolysis

Glycolysis rates were determined by perfusing hearts with [5-^{3}H]glucose. Total myocardial ^3H_2O production liberated from [5-^{3}H]glucose at the enolase step of glycolysis was determined every 10 minutes. The rates expressed as µmol/gdw·min were calculated for each time interval. Averaged values were calculated for the 30-minute perfusion without insulin and for the subsequent 60 minutes with insulin.

Glycogen Content and [5-^{3}H]Glucose Incorporation

Glycogen content (in µmol glucosyl units/gdw) was determined by powdering heart tissue samples and subjecting to alkaline extraction with 30% potassium hydroxide (KOH) followed by ethanol precipitation and acid hydrolysis (2 N H_2SO_4) and analysis of glucose content. Incorporation of [5-^{3}H]glucose into glycogen was counted.[6]

Glucose Uptake in Perfused Working Hearts

Glucose uptake (in µmol glucose/gdw·min) was calculated taking into account the 2 fates of glucose subjected to intermediary metabolism, that is, metabolism by glycolysis and incorporation into glycogen.[6] The average rate of glucose uptake in the time interval 30–90 minutes ($GU_{[30–90]}$) (treatment period) is the sum of the rate of glycolysis and the rate of glycogen synthesis (abbreviated as $GG_{[30–90]}$):

$$GU_{(30–90)}\left[\text{mmol}\times\text{g}^{-1}\times\text{minute}^{-1}\right]=$$
$$\text{glycolysis}_{(30–90)}\left[\text{mmol}\times\text{g}^{-1}\times\text{minute}^{-1}\right]+$$
$$GG_{(30–90)}\left[\text{mmol}\times\text{g}^{-1}\times\text{minute}^{-1}\right]$$

Glycogen synthesis in the time interval 30–90 minutes (treatment period; $GG_{[30–90]}$) is calculated from the total amount of newly synthesized glycogen measured at the end of the protocol (90 minutes) and the average rate of glycogen synthesis in the first 30 minutes ($GG_{[0–30]}$) determined from separate experiments:

$$\text{"Hot" glycogen content}\left[\text{mmol}\times\text{g}^{-1}\right]=$$
$$GG_{(0–30)}\left[\text{mmol}\times\text{g}^{-1}\times\text{minute}^{-1}\right]\times 30\text{ minutes}+$$
$$GG_{(30–90)}\left[\text{mmol}\times\text{g}^{-1}\times\text{minute}^{-1}\right]\times 60\text{ minutes}$$

Glycolytic Metabolites, Citrate Concentrations, Pyruvate Dehydrogenase Activity, and Nuclear Factor κB Activation

Glucose 6-phosphate, fructose 6-phosphate (F6P), and fructose 1,6-bisphosphate (F1,6BP) levels (nmol/g wet

weight) were determined by a coupled enzymatic assay after deproteinizing samples with perchloric acid.[17] Citrate was determined from heart tissue homogenate using a commercially available kit (Abcam [Cambridge, MA] #ab83396) according to manufacturer's instructions. Nuclear factor κB (NFκB) (p65) deoxyribonucleic acid (DNA)-binding activity was assayed using nuclear fractions prepared as recommended by the manufacturer (Thermo Scientific, Oakville, ON). Pyruvate dehydrogenase (PDH) activity (mOD/min/mg) was measured using commercially available kits (Abcam #ab109902).

Immunoblotting

Total tissue lysate was prepared from frozen cardiac samples in ice-cold lysis buffer containing 50 mM Tris (pH, 8.0), 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.1% sodium dodecyl sulfate (SDS), and 0.5% sodium deoxycholate (supplemented with protease and phosphatase inhibitor cocktail mix), and the supernatant after centrifugation at 1000*g* (10 minutes, 4°C) was collected. Plasma membrane–enriched fractions were prepared.[18] Protein concentrations were quantified by Bradford assay and boiled in 5× SDS-loading buffer (except for plasma membrane fractions). Equal amount of protein samples (20–25 μg) was loaded, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane for detection with antibodies of interest. Immunoreactivity was visualized using an enhanced chemiluminescence (ECL) reagent (GE HealthCare, Mississauga, ON, Canada), and its intensity was quantified using ImageJ software (http://rsbweb.nih.gov/ij/). The following antibodies were from (1) Cell Signaling Technology (Danvers, MA): p-Akt (Ser473 and Thr308; #9271 and #9272), total Akt (#9272), p-AMPKα (Thr172; #2531), total AMPKα (#2532), p-GSK3β (Ser9; #9336), total glycogen synthase kinase 3β (GSK3β) (#9315); (2) Abcam Inc (Cambridge, MA): CD36 (#ab133625), phosphoinositide-dependent protein kinase (PDK)-4 (#ab214938), p-p70S6K1 (Thr389; #ab2571), total p70S6K1 (#ab9366), α-tubulin (#ab7291), vinculin (#ab18058); (3) Santa Cruz Biotechnology (Dallas, TX): p-PFK2 car (Ser466 and Ser483; #sc32966 and #sc373806), p-PP2A-C α/β (Tyr307, #sc271903), PP2A-C (#sc80665), demethylated-PP2A-C (#sc13601), PP2A-B55α (#sc81606); (4) Novus Biologicals (Littleton, CO): GLUT4 (#NBP1-49840), Na^+/K^+-ATPase α1 (#NB300-146SS); (5) Aviva Systems Biology (San Diego, CA): PFK2 (#ARP56677); and (6) Sigma-Aldrich (St Louis, MO): α-tubulin (#T6074). Loading controls (α-tubulin, vinculin, Na^+/K^+-ATPase α1) were used where appropriate. Because no satisfying blotting conditions could be established for both antibodies phospho- and total PFK2 on the same membrane, phospho-to-total PFK2 ratios were determined from separate blots for p-PFK2 and total PFK2, each normalized to the respective loading controls, to obtain high-quality signals.

Acylcarnitine Profiling of Cardiac Tissue Using Mass Spectrometry

Tissue levels of 32 acylcarnitine species were measured using electrospray ionization tandem mass spectrometry. Acylcarnitines were extracted from heart tissue with methanol and quantified using 8 isotopically labeled internal standards (Cambridge Isotopes Laboratories, Andover, MA). Precursor ions of m/z 85 in the mass range of m/z 150–450 were acquired on a PE SCIEX API 365 LC-ESI-MS/MS instrument (Applied Biosystems, Foster City, CA).[6,19]

Statistical Analysis

Values are given as mean (standard deviation [SD]) or median (25th–75th percentile) depending on the underlying data distribution (normal versus skewed) for the indicated number of independent observations (N). The significance of differences in metabolic variables (such as glucose uptake, glycogen levels, and protein phosphorylation levels) among groups was determined by ANOVA followed by the Tukey method for post hoc analysis or by nonparametric methods (Kruskal–Wallis test followed by Dunn test for post hoc analysis) depending on the underlying data distribution. Two-way repeated-measures analysis of variance (ANOVA) was used to analyze glycolysis data as well as hemodynamic data. This was followed by all pairwise multiple comparison procedures (Holm–Sidak method) if appropriate to determine which group differences were statistically significant. Differences are considered statistically significant if the overall $P < .05$ (2-sided). SigmaPlot (version 13.0; Systat Software, Inc, Chicago, IL) was used for the analyses. A sample size estimate was calculated based on our previous results.[6] With an expected difference in the main independent variable (glucose uptake) of 1 μmol·gram dry heart weight $[gdw]^{-1}$·$minute^{-1}$, a SD of 0.5 μmol·gdw^{-1}·$minute^{-1}$, an α level of .05 (2-sided), a power of 0.8, and 4 groups (no-INS, INS, INS/IL, and INS/OV; Figure 1) in a 1-way ANOVA design, a minimum sample size of N = 7 hearts per group was calculated.

RESULTS

Cardiac Performance Remains Stable in the Presence of the Lipid Emulsions Omegaven and Intralipid

A summary of the hemodynamic measurements is given in Supplemental Digital Content, Table 2, http://links.lww.com/AA/C857. LVW and CO were unchanged after insulin administration. Administration of Intralipid or Omegaven did not

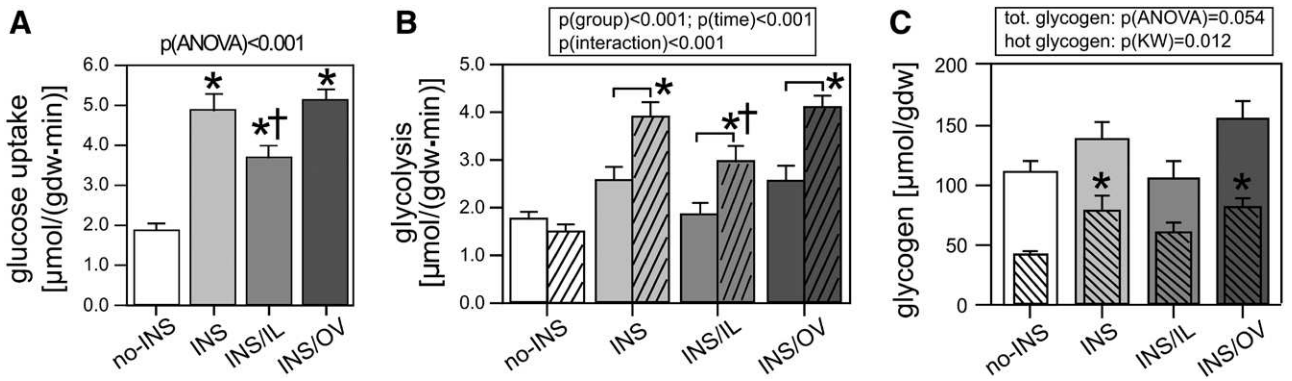

Figure 2. Glucose uptake, glycolysis, and glycogen metabolism. A, Glucose uptake was calculated from glycolytic flux and glycogen synthesis over 60 min in all groups. B, Average rates of glycolysis without insulin (first 30 min, open bars) and with insulin (subsequent 60 min, hatched bars). Horizontal brackets indicate significant differences for factor "time." C, Total and radiolabeled (hatched bars) glycogen tissue content at the end of perfusions. N = 7 for each group. *Significantly different from no-INS group. †Significantly different from both the INS and INS/OV groups. Data are mean (SE). ANOVA indicates analysis of variance; gdw, gram dry heart weight; INS, insulin alone; INS/IL, perfusion with insulin plus Intralipid; INS/OV, perfusion with insulin plus Omegaven; KW, Kruskal-Wallis; no-INS, perfusion without insulin; SE, standard error.

affect any of the measured parameters including heart rate, coronary flow, CO, and LVW.

Omegaven as Opposed to Intralipid Preserves Glycolysis, Glycogen Synthesis, and Glucose Uptake

Administration of insulin to the perfusate, containing 11 mM glucose and 1.2 mM palmitate bound to albumin, for 60 minutes after 30 minutes of perfusion without insulin increased glycolysis (INS baseline 2.58 ± 0.28 µmol/gdw·min versus INS response 3.91 ± 0.30 µmol/gdw·min; $P < .001$), while glycolysis did not change without insulin administration (no-INS baseline 1.77 ± 0.15 µmol/gdw·min versus no-INS response 1.51 ± 0.15 µmol/gdw·min; $P = .27$). Administration of insulin also increased incorporation of radioactive glucose into glycogen (INS 73.4 µmol/gdw [42.0–112.4 µmol/gdw] versus no-INS 41.4 µmol/gdw [38.5–44.6 µmol/gdw]; $P = .042$) and glucose uptake (INS 4.9 ± 0.4 µmol/gdw·min versus no-INS 1.9 ± 0.2 µmol/gdw·min; $P < .001$) (Figure 2A–C). Intralipid administration markedly diminished the insulin response, as evidenced by reduced glycolysis (INS/IL response 3.03 ± 0.3 µmol/gdw·min versus INS response 3.91 ± 0.30 µmol/gdw·min; $P = .038$) and reduced net glucose uptake (INS/IL 3.7 ± 0.3 µmol/gdw·min versus INS 4.9 ± 0.4 µmol/gdw·min; $P = .047$). In contrast, Omegaven did not reduce glycolysis (INS/OV response 4.11 ± 0.24 µmol/gdw·min versus INS response 3.91 ± 0.30 µmol/gdw·min; $P = .59$) and fully preserved glucose uptake (INS/OV 5.1 ± 0.3 µmol/gdw·min versus INS 4.9 ± 0.4 µmol/gdw·min; $P = .94$) (Figure 2A–C). Consistent with fatty acid–induced inhibition of glucose oxidation (Randle cycle), Intralipid reduced PDH activity (INS/IL 18.7 mOD/min/mg [15.3–21.0 mOD/min/mg] versus INS 31.7 mOD/min/mg [22.0–40.8 mOD/min/mg]; $P = .019$) (Figure 3A), while PDK4 protein levels were unchanged (Figure 3B). Conversely, administration of Omegaven did not reduce PDH activity (INS/OV 24.6 mOD/min/mg [23.8–36.5 mOD/min/mg] versus INS 31.7 mOD/min/mg [22.0–40.8 mOD/min/mg]; $P = 1.000$). Translocation of GLUT4 to the sarcolemma in response to insulin was unaffected in Intralipid- or Omegaven-treated hearts (Figure 3C). There was a trend to higher GSK3β phosphorylation in Omegaven-treated hearts, but this did not reach statistical significance (Figure 3D). Analysis of glycolytic intermediates (Supplemental Digital Content, Table 3, http://links.lww.com/AA/C857) revealed reduced F1,6BP-to-F6P ratio in Intralipid-treated hearts (INS/IL 0.47 ± 0.09 versus INS 0.59 ± 0.04; $P = .035$), but not in Omegaven-treated hearts (INS/OV 0.60 ± 0.11 versus INS 0.59 ± 0.04; $P = .98$), consistent with decreased activity of PFK-1 (Figure 3E). Tissue concentrations of citrate, an allosteric regulator of PFK-1, were not different between groups (Figure 3F). Together, these data show reduced glucose uptake as a result of impaired glycolysis and glycogen synthesis in Intralipid-treated hearts, while Omegaven-treated hearts show unchanged glucose uptake because of preserved glycolytic flux.

Omegaven Inhibits PP2A and Enhances Phosphorylation of Akt_{T308}, $p70S6K_{T389}$, and $PFK\text{-}2_{S483}$

Based on our hypothesis that inhibition of PP2A activity would be involved in the observed beneficial effects of Omegaven on glycolytic flux, we determined PP2A phosphorylation and its methylation status, because both are known to affect PP2A activity.[20,21] Omegaven increased phosphorylation at Y307, a phosphorylation site by receptor tyrosine kinases and Src kinase family members,[20] compared to Intralipid treatment or insulin treatment alone (Figure 4A). The demethylation status of PP2A was unaffected (Supplemental Digital

Content, Figure 1A, http://links.lww.com/AA/C857). Increased phosphorylation of PP2A was accompanied by increased Akt_{T308} and $p70S6K_{T389}$ phosphorylation (Figure 4B, C). No changes in Akt_{S473} were observed (Figure 4D). PFK2, the downstream target of PDK1/Akt signaling, did not show changes in phosphorylation at S466 with Intralipid or Omegaven (Figure 4E), but there was an increased phosphorylation at S483 with Omegaven, indicating enhanced PFK2 activity (Figure 4E). Phosphorylation of AMPK was not affected by Intralipid or Omegaven (Supplemental Digital Content, Figure 1B, http://links.lww.com/AA/C857).

Omegaven but Not Intralipid Causes Accumulation of Acylcarnitines and NFκB Activation

To determine the effect of the lipid emulsions Intralipid and Omegaven on cellular fatty acid uptake and β-oxidation, we established the acylcarnitine profiles (Supplemental Digital Content, Table 4, http://links.lww.com/AA/C857). Omegaven-treated hearts showed elevated free carnitine levels without decrease in acetylcarnitine, consistent with reduced cellular fatty acid uptake compared with the other groups (Figure 5A). CD36 in the membrane fraction was not different between groups (Supplemental Digital Content, Figure 1C, http://links.lww.com/AA/C857). There was accumulation of most other acylcarnitine species. Short-, medium-, and to a lesser degree, long-chain acylcarnitines (mainly C16:1, C18:1, and long-chain hydroxyl [OH]) were increased in Omegaven- but not Intralipid-treated hearts (Figure 5B–E), consistent with decelerated β-oxidation (Supplemental Digital Content, Table 3, http://links.lww.com/AA/C857). The acylcarnitine profile also showed a reduction in the low-abundance

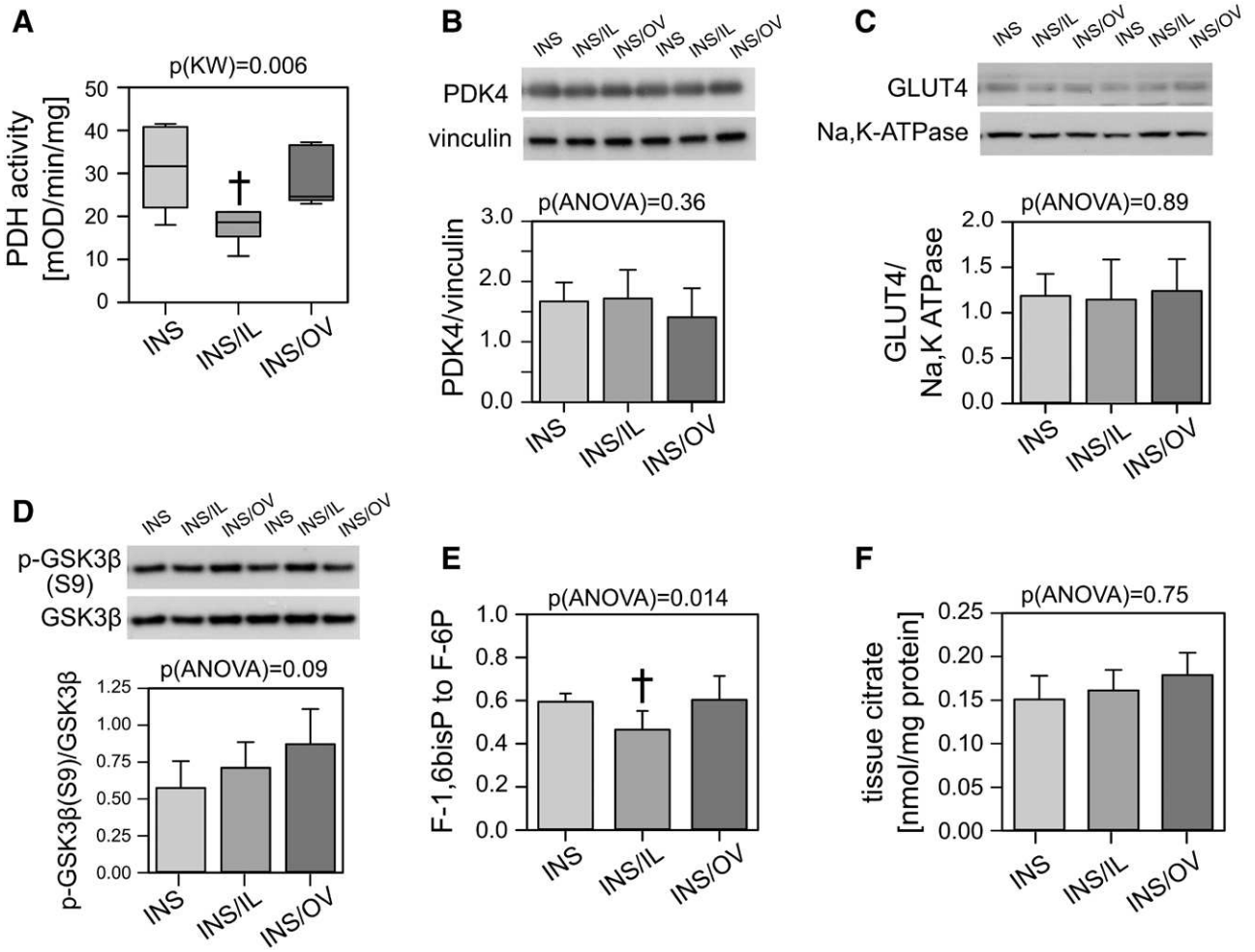

Figure 3. Key regulators of cardiac glucose metabolism. A, PDH activity. B, PDK4 expression normalized to vinculin. C, GLUT4 in membrane fractions normalized to Na^+/K^+-ATPase. D, Phosphorylation of GSK3β normalized to total GSK3β. E, Phosphofructokinase-1 activity as measured by the ratio between glycolytic metabolites F-1,6bisP and F-6P. F, Citrate tissue concentrations. N = 7 for each group. †Significantly different from both the INS and INS/OV groups. Data are mean (SD). ANOVA indicates analysis of variance; F-1,6bisP, fructose 1,6-bisphosphate; F-6P, fructose 6-phosphate; GLUT4, glucose transporter type 4; GSK3β, glycogen synthase kinase 3β; INS, insulin alone; INS/IL, perfusion with insulin plus Intralipid; INS/OV, perfusion with insulin plus Omegaven; KW, Kruskal-Wallis; Na^+/K^+-ATPase, sodium-potassium adenosine triphosphatase; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; SD, standard deviation.

C18:2 species exclusively in Intralipid-treated hearts, consistent with an increase in β-oxidation, because this low-abundance acylcarnitine species is specifically sensitive to flux changes in β-oxidation because it is poorly cleaved from triglycerides by lipoprotein lipase.[22] To evaluate whether the decelerated β-oxidation and accumulation of acylcarnitines in Omegaven-treated hearts were associated with a higher reactive oxygen species (ROS) release from mitochondria,[19,23] we measured the activation of the ROS-sensitive biomarker NFκB.[24] Indeed, Omegaven was associated with higher NFκB activation when compared with insulin-treatment alone (INS/OV 5.0 ± 0.9 absorbance/mg versus INS 3.5 ± 1.3 absorbance/mg; P = .038) (Figure 5F). This observation suggests that a decelerated β-oxidation accompanied by accumulation of acylcarnitines causing a prooxidant environment may be involved in inhibiting the redox-sensitive PP2A and preserving the phosphorylation status of key insulin signaling proteins.

DISCUSSION

A recent study showed that acute exposure of cardiomyocytes to marine n3 fatty acids prevented insulin resistance.[13] But it is not clear so far whether acute exposure of n3 as opposed to n6 fatty acids has beneficial effects on glucose metabolism in the intact beating heart, and if so, what the underlying mechanisms could be. We have addressed these questions by using isolated perfused rat hearts under standardized conditions of work load, insulin challenge, and energy substrate concentrations, comparing the effects of Omegaven versus Intralipid on glucose metabolism. Our study shows the following salient findings. The use of the n3 fatty acid–based lipid emulsion Omegaven as opposed to Intralipid fully preserved

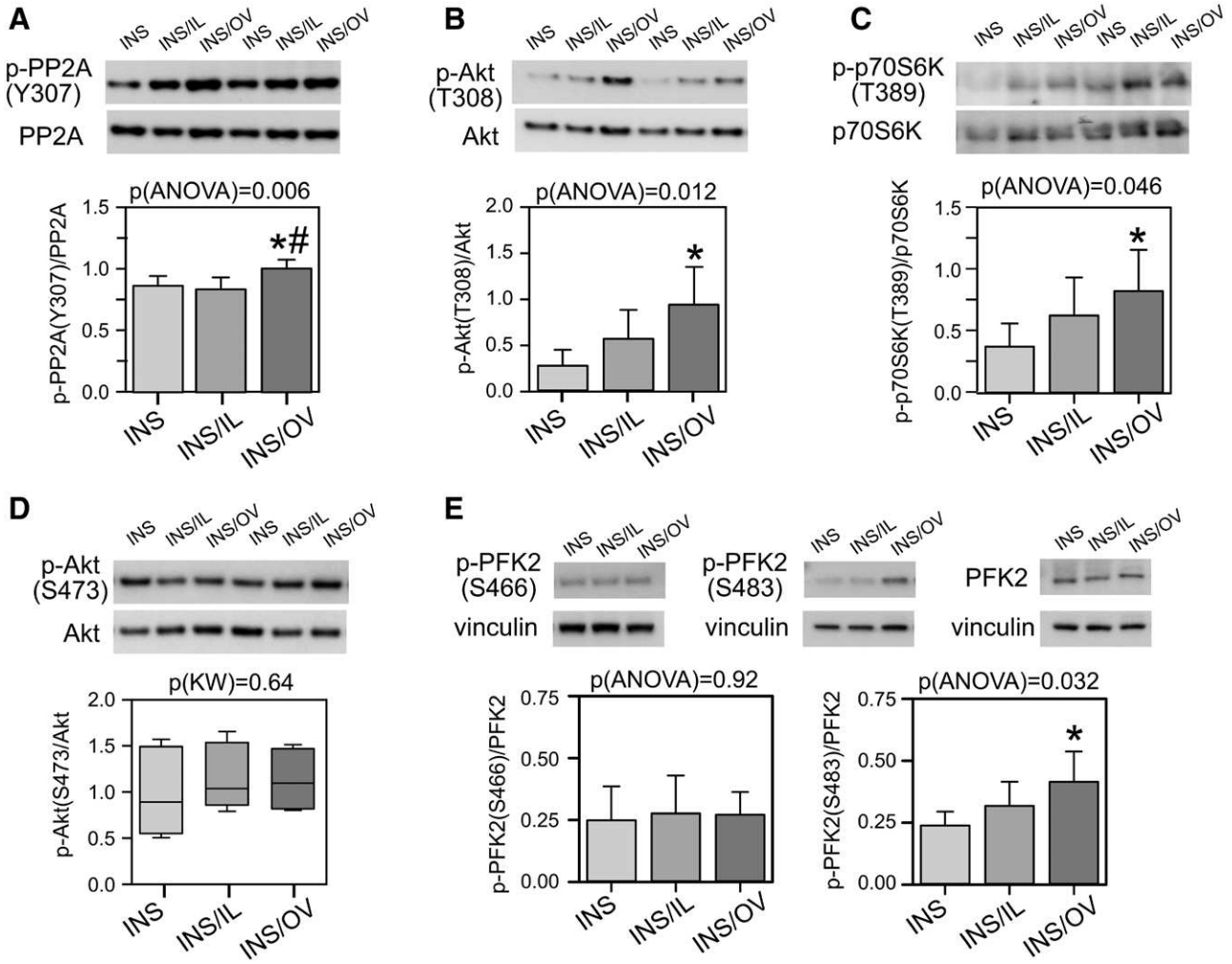

Figure 4. Serine/threonine PP2A and phosphorylation status of key insulin signaling proteins. A, Phosphorylation (Y307) of PP2A normalized to total PP2A. Increased phosphorylation at Y307 indicates inhibition. B, Phosphorylation (T308) of Akt normalized to total Akt. C, Phosphorylation of p70S6K (T389) to total p70S6K. D, Phosphorylation (S473) of Akt normalized to total Akt. E, Phosphorylation of PFK2 (S466, S483) normalized to total PFK2. Immunoblots of phospho- and total PFK2 were first separately normalized to vinculin (see Methods). N = 7 for each group. *Significantly different from INS group. #Significantly different from INS/IL group. Data are mean (SD). ANOVA indicates analysis of variance; INS, insulin alone; INS/IL, perfusion with insulin plus Intralipid; INS/OV, perfusion with insulin plus Omegaven; KW, Kruskal-Wallis; p70S6K, p70S6 kinase; PFK2, phosphofructokinase-2; PP2A, protein phosphatase 2A; SD, standard deviation.

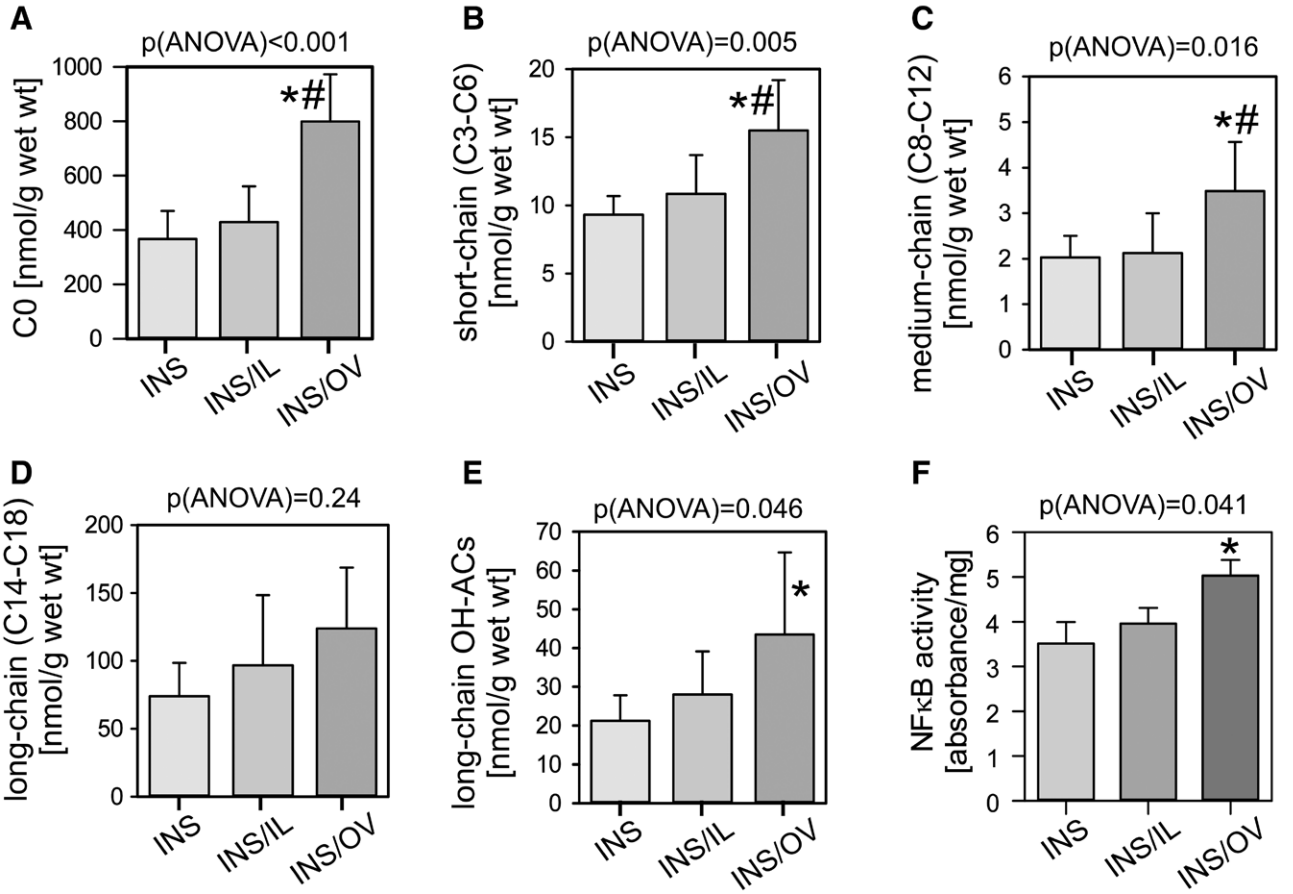

Figure 5. Acylcarnitine tissue profiles. A, Free carnitine tissue levels (C0). B, Short-chain (C3–C6) acylcarnitine tissue levels. C, Medium-chain (C8–C12) acylcarnitine tissue levels. D, Long-chain (C14–C18) acylcarnitine tissue levels. E, OH-ACs tissue levels. F, NFkB DNA-binding activity in cardiac tissues measured from nuclear extracts. N = 7 for each group. *Significantly different from INS group. #Significantly different from INS/IL group. Data are mean (SD). ANOVA indicates analysis of variance; DNA, deoxyribonucleic acid; INS, insulin alone; INS/IL, perfusion with insulin plus Intralipid; INS/OV, perfusion with insulin plus Omegaven; NFkB, nuclear factor-kB; OH-AC, long-chain hydroxyl acylcarnitine; SD, standard deviation.

the metabolic response to insulin and maintained glycolysis, glycogen synthesis, and glucose uptake. Omegaven-mediated preserved glucose uptake was associated with preserved PFK1 activity, the rate-limiting enzyme of glycolysis. The presence of Omegaven enhanced phosphorylation of the key insulin signaling proteins Akt_{T308}, but not Akt_{S473}, $p70S6K_{T389}$, and PFK-2 ($PFK2_{S483}$), consistent with PP2A inhibition (Figure 6). Activated PFK2 produces more fructose 2,6-bisphosphate (F2,6BP), the key allosteric activator of PFK1, which offsets effects by the Randle cycle, that is, inhibition of glycolysis by fatty acids.[9] Our experiments have further explored possible mechanisms underlying Omegaven-mediated PP2A inhibition and pinpoint to a potential role of a decelerated β-oxidation with accumulation of acylcarnitine species fostering a prooxidant cellular environment that potentially inhibits redox-sensitive PP2A[21] enhancing insulin signaling.[14,20]

Glycolysis is regulated by insulin through activation of PFK2, the enzyme-synthetizing F2,6BP, which is a potent activator of PFK1, the rate-limiting enzyme of glycolysis. Although PFK2 was previously recognized as an Akt substrate and found to be phosphorylated and activated by Akt at S466 and S483, more recent research pinpoints PDK1 rather than Akt as the upstream kinase, where S466 phosphorylation is thought to increase the maximum rate of reaction (V_{max}) and S483 phosphorylation is thought to reduce citrate inhibition.[25] Consistent with this concept is the observation that insulin fails to stimulate PFK2 in PDK1-deficient mice in vivo.[26] Our own results from Omegaven-treated rat hearts show a selective phosphorylation of Akt_{Thr308} and phosphorylation of PFK2 at S483 (but not S466) in accordance with observations in insulin-treated rat cardiomyocytes.[27] We also found phosphorylation and activation of p70S6K,[28] which reportedly reduces PFK2 protein degradation via autophagy.[29] Our studies in intact beating hearts demonstrate that modulation of PFK2 phosphorylation by Omegaven is involved in the preservation of glycolytic flux and glycogen synthesis (most likely allosterically by glucose itself[30]) despite the presence of palmitate in the perfusate and additional exposure to fatty acids released from Omegaven.

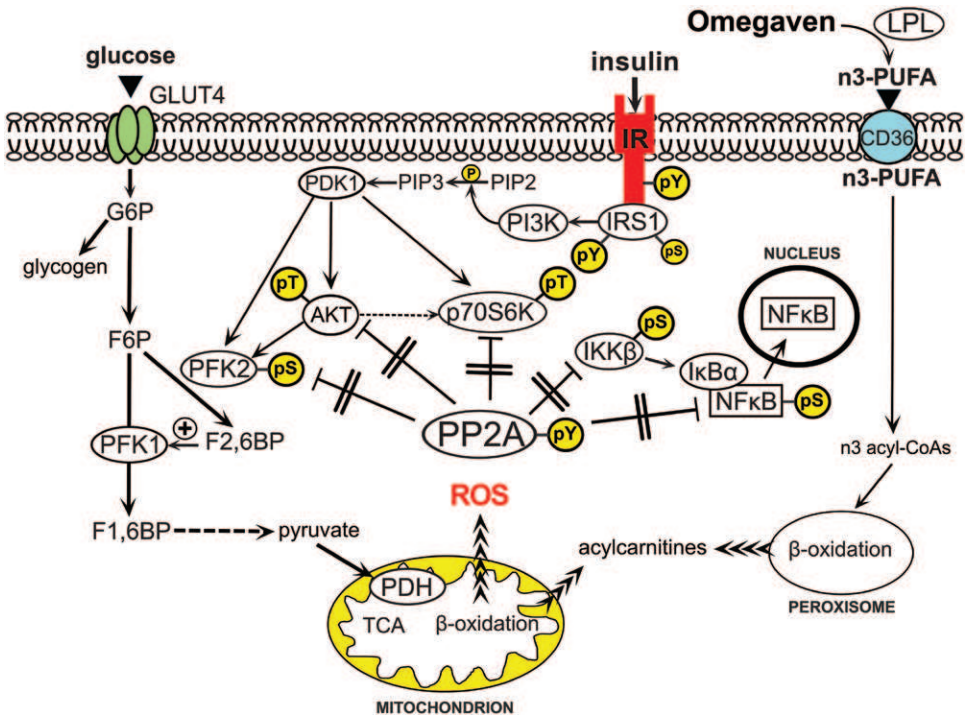

Figure 6. Working model of n3 fatty acid–induced preservation of insulin signaling and glucose uptake in the intact heart. Release of n3-PUFAs including EPA (C20:5 n3) and DHA (C22:6 n3) by LPL from Omegaven and their subsequent degradation in peroxisomes and mitochondria leads to accumulation of acylcarnitine species causing formation of ROS and inhibition of the redox-sensitive serine/threonine PP2A. Preservation of the phosphorylation status of its targets Akt (at Thr308), PFK2, which in turn activates PFK1 via its product fructose 2,6-bisphosphate, and p70S6K, which leads to reduced ubiquitination of PFK2, maintains glycolytic flux in response to insulin stimulation. PDK1 is possibly an additional target of PP2A. NFκB signaling is also affected by PP2A and ROS. CD36 indicates cluster of differentiation 36; DHA, docosahexaenoic; EPA, eicosapentaenoic; F1,6BP, fructose 1,6-bisphosphate; F2,6BP, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; GLUT4, glucose transporter type 4; IκBα, nuclear factor of κ light polypeptide gene enhancer in B cell inhibitor α; IKκβ, inhibitor of nuclear factor κB kinase subunit β; IR, insulin receptor; IRS1, insulin receptor substrate 1; LPL, lipoprotein lipase; n3 acyl-CoAs, n3 fatty acyl-CoA thioesters; n3-PUFA, n3 polyunsaturated fatty acids; NFκB, nuclear factor κB; p70S6K, p70S6 kinase; PDH, pyruvate dehydrogenase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PFK1, phosphofructokinase-1; PFK2, phosphofructokinase-2; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PP2A, protein phosphatase 2A; pS, phosphorylated serine residue; pT, phosphorylated threonine residue; pY, phosphorylated tyrosine residue; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

PP2A accounts for 80% of total serine/threonine phosphatase activity and represents a major proportion of protein phosphatases in mammalian cells.[21] PP2A specifically dephosphorylates Akt_{T308},[31] PFK2,[32] and p70S6K,[28] and possibly PDK1.[33] PP2A is inhibited by phosphorylation at Y307 by receptor tyrosine kinases including the insulin receptor,[20] but also has the capability to reactivate itself by auto-dephosphorylation.[21] In our study, inhibition of PP2A could be mediated by activation of Src,[20] which phosphorylates and inhibits PP2A at Y307. In fact, EPA and DHA, n3 fatty acids released from Omegaven, have been shown to activate Src in a redox-sensitive manner, which subsequently enhances Akt signaling in cultured coronary endothelial cells.[34] Alternatively, PP2A could be directly inhibited by ROS,[23] which could impair autodephosphorylation and its reactivation. There was no inhibition of PP2A as evidenced by phosphorylation at Y307 in the Intralipid group and the small changes in phosphorylation at Akt (T308) and p70S6K (T389) in this group may be due to the overall changed metabolic conditions in the presence of additional fatty acids. Together, our findings highlight the potential role of PP2A inhibition in n3 fatty acid–induced modulation and preservation of insulin signaling and glucose metabolism in the intact beating heart.

We reasoned that the different fatty acids released by lipoprotein lipase from the 2 lipid emulsions Intralipid and Omegaven would elicit a characteristic acylcarnitine profile, which could help understand the effects of Omegaven on glucose metabolism. The acylcarnitine profiles showed 2 major findings: (1) Omegaven decreased the uptake of fatty acids into cardiomyocytes, as evidenced by higher free carnitine levels, in the presence of unchanged acetylcarnitine and without reducing CD36 in the sarcolemma. This is in line with previous studies showing that long-chain n3 fatty acids, specifically EPA and DHA, inhibit the hydrolysis of triglyceride emulsions. The presence of >20% n3 fatty acids inhibited hydrolysis by 50%, and Omegaven contains >40% of EPA and DHA. Levy and Herzog[22] have meticulously assessed the hydrolysis of individual fatty acids of chylomicron triacylglycerol by cardiac lipoprotein lipase and

found that the average mobilization of a long-chain fatty acid within 60 minutes is ≈25% as opposed to EPA with only 6% and DHA with only 2% mobilization, respectively. In addition, as EPA and DHA are also transported via CD36, the main transporter of fatty acids into cardiomyocytes, the sterically curved polyunsaturated fatty acids as opposed to straight linear saturated fatty acids such as palmitate could impair the CD36 transport mechanism and reduce the overall uptake.[35] It is therefore possible that a decrease in fatty acid uptake may have contributed to the reduced inhibition of glycolysis through lower citrate tissue concentrations, that is, the Randle cycle, but we did not observe decreased citrate tissue levels in Omegaven-treated hearts. However, small changes in compartmentalized citrate concentrations may be difficult to detect. (2) Omegaven-treated hearts showed accumulation of acylcarnitines, consistent with decelerated β-oxidation. While peroxisomes are the major site of degradation of very long chain and unsaturated fatty acids, most degradation steps of fatty acids also occur in mitochondria.[36] The degradation of n3 polyunsaturated fatty acids imposes a substantial metabolic burden on β-oxidation. As double bonds in naturally occurring fatty acids are *cis*, but the β-oxidation can only handle *trans* double bonds at the enoyl-CoA hydratase step, 2 additional enzymes are required, namely a *cis-trans* isomerase, and an epimerase.[36] These additional steps slow down β-oxidation and may lead to significant accumulation of acylcarnitine species, as observed in Omegaven-treated hearts. Although increased β-oxidation intermediates can cause mitochondrial stress and are often considered a hallmark of the diabetic metabolism,[23] some studies report an improvement of glucose oxidation with n3 fatty acid–induced acylcarnitines accumulation.[12] Because previous studies reported ROS formation and activation of NFκB in response to acylcarnitine species,[37] we tested the activation of NFκB as ROS biosensor and found that Omegaven- as opposed to Intralipid-treated hearts showed NFκB activation. Rutkowsky et al[37] showed ROS production by C14 acylcarnitines from nonmitochondrial sources with concomitant NFκB activation in RAW264.7 cells. We have also shown ROS formation directly from mitochondria in response to palmitoylcarnitine, possibly via inhibition of the electron transport chain.[19] Interestingly, ROS-mediated PP2A inhibition is also known to activate NFκB in an nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitor α (IκBα)-dependent[38] and independent manner.[39] Irrespectively, the cellular prooxidant environment and the accumulation of β-oxidation intermediates activated NFκB as observed in our own experiments.

Potential Clinical Implications

Lipid emulsions are clinically used for parenteral nutrition in critically ill patients. They form an important part of all parenteral nutrition formulations because they provide essential fatty acids and the necessary density of calories. However, they also elicit insulin resistance,[10,11] which is known to cause adverse clinical outcomes.[5] Although we did not observe reduced cardiac function in our study of healthy perfused rat hearts under aerobic conditions, it is likely that insulin resistance may cause further harm to the already stressed critically ill patient and reduce cardiac function and tolerance against ischemia–reperfusion injury.[40,41]

Limitations of the Study

We did not directly measure F2,6BP in cardiac tissue, but our experiments imply that the increase in PFK1 activity is due to higher amounts of its key allosteric activator F2,6BP, generated by the phosphorylated and activated PFK2. Unfortunately, F2,6BP is only present in very low tissue concentrations and subject to great instability and thus cannot be measured by standard metabolic approaches. Additional experiments will be necessary to ultimately prove that ROS formation is indeed the actual cause of the preserved insulin signaling in hearts treated with n3 fatty acids. These experiments should also determine the source of ROS production (NADPH oxidases versus electron transport chain). Although inhibition and not activation of NFκB have been shown in other noncardiac tissues, mainly inflammatory cells, in response to n3 fatty acids, our observation in the heart that acute exposure to n3 fatty acids elicits NFκB signaling is still compatible with beneficial effects because NFκB signaling is also known to play a role in cardioprotection.[42] It will be important to determine whether Omegaven is also able to preserve glucose metabolism in diseased lipid-overloaded diabetic hearts. Finally, although the exact compositions of the soybean oil–derived Intralipid and the fish oil–derived Omegaven may slightly vary, the compositions of these 2 commercially available lipid emulsions are usually very consistent and stable. Nonetheless, there may be additional less well-characterized compounds in the 2 emulsions, which could theoretically account for some of the observed differences in insulin signaling and metabolism. However, a myriad of previous studies identified the difference in the released fatty acid species (specifically the ratio between n6 and n3 fatty acids) as the major cause of differences in metabolic outcomes when comparing these 2 lipid emulsions.

In summary, our study addressed an important knowledge gap whether differences in the composition of lipid emulsions lead to differing degrees of insulin sensitivity in the heart. Using isolated perfused hearts, we show that Omegaven as opposed

to Intralipid preserves glucose uptake via the PP2A–Akt–PFK pathway. The presence of n3 fatty acids appears to decelerate β-oxidation causing acylcarnitine species accumulation and a prooxidant response, potentially inhibiting redox-sensitive PP2A and preserving the phosphorylation state of key insulin signaling proteins and ultimately glucose uptake.

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DISCLOSURES

Name: Phing-How Lou, PhD.
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Name: Martin Hersberger, PhD.
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Contribution: This author helped design the study, interpret the data, write the manuscript, and approve the final manuscript.
Name: Michael Zaugg, MD, MBA, FRCPC.
Contribution: This author helped design the study, interpret the data, write the manuscript, and approve the final manuscript.
This manuscript was handled by: Alexander Zarbock, MD.

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